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Breast cancer progression may be characterized by a switch from hormone-dependent to hormone-independent growth that involves several cellular alterations and is a major problem in the treatment of breast cancer. Expression of a constitutively activated Raf in ER+ MCF-7 human breast cancer cells results in estrogen-independent growth, suggesting that activation of growth factor signaling pathways through Raf may confer a selective advantage for growth of breast cancer cells under estrogen-deprived conditions. In analyzing the mechanisms underlying this, it was discovered that the prolonged growth of these cells in the absence of estrogen also resulted in loss of ER expression. The work presented here has focused on determining the mechanisms underlying both the estrogen-independent growth and loss of ER expression. We have determined that constitutive MEK activity results in a similar phenotype. We have also determined that loss of ER expression is correlated with changes in transcription factor binding to the ER promoter, preferential ERK 2 activity, and extent of MAPK activity. We have also correlated our Raf results with the effects of EGFR or c-erbB-2 overexpression in MCF-7 cells. Finally, we have demonstrated that the abrogation of Raf signaling allows for re-expression of ER.

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Donna O. Ashley 3/5/99

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INTRODUCTION

Clinically, breast cancer presents as either estrogen receptor (ER) positive or as ER negative. The presence of estrogen receptor is correlated with a better prognosis both in terms of increased disease-free survival and overall survival, and predicts for response to hormonal therapies such as tamoxifen (1-4) ER+ tumors can progress to ER- tumors which are characterized by a more aggressive phenotype, a poor prognosis, and a lack of response to hormonal therapies. Steps in this pathway may include the constitutive expression of growth promoting genes once regulated by estrogen leading to a loss of estrogen dependence, an acquired resistance to antiestrogens, the loss of expression of ER, and an acquisition of a more aggressive phenotype. In fact, many growth factor receptors are overexpressed in breast tumors. Two such receptors that are up-regulated in ER- tumors are the epidermal growth factor receptor (EGFR) and c-erbB-2; and they are also important prognostic indicators. For example, in breast cancer cells, the expression of EGFR is inversely correlated with ER: the majority of breast tumors are either ER+/EGFR- or ER-/EGFR+ (5), and EGFR+ tumors have a poor prognosis independent of ER status (6-10). Similarly, tumors that overexpress c-erbB-2 have a poorer prognosis and tend to be ER- (11-13). The protooncogene Ras, the downstream mediator of growth factor receptor activation, is overexpressed in ~70% of breast cancer (14,15), further implicating growth factor signaling mechanisms in breast cancer. Double-label immunohistochemical detection of ER and EGFR in breast tumor specimens and breast cancer cell lines confirms the inverse correlation of expression (16-18). Furthermore, in ER+/EGFR+ tumors, individual tumor cells express high levels of only ER or EGFR, but never both (16,17). The

EGFR+ cells in these tumors are also associated with a higher growth rate than the ER+ cells (19,20). Interestingly, ER and EGFR expression in the same cell is observed in normal and bengin breast specimens (17), suggesting that interaction between these two signaling pathways is altered in breast cancer cells. Stable transfections of growth factor signaling components into ER+ MCF-7 breast cancer cells, i.e. EGFR, erbB-2, HRG, or Ras, leads to decreased but not completely absent levels of ER in most cases, in addition to estrogen-independent growth (21-26). For example, overexpression of c-erbB-2 in MCF-7 cells by two different groups resulted in an approximate 50% decrease in ER levels in one case (23), and in an approximate 4-fold reduction in ER in one clone with a constitutively activated erbB-2 in the other case (22). We have established a model of up-regulated growth factor signaling via Raf-1 by stably expressing a constitutively active Raf-1 in MCF-7 cells and have demonstrated that this confers on the cells estrogen-independence (27). In addition, however, high levels of Raf-1 activity completely down-regulate ER expression. Thus, our system of chronic activation of a downstream growth signaling pathway resulting in both estrogen-independent growth and loss of ER implicates the activation of Raf as the mediator of signal transduction induced down-regulation of ER. Therefore, it seems imperative to dissect the mechanisms underlying the role of growth factor signaling in the progression from ER+ to ER- in order to attempt to establish common targets for inhibition to allow for re-expression of ER

The estrogen receptor is part of a large family of nuclear receptors that act as hormone-inducible transcription factors and share a common structure [reviewed in (28-30)]. Binding of hormone activates receptor to the tight DNA binding state which involves, among many other events, phosphorylation and the acquisition of the ability to recognize and bind hormone

responsive elements (HREs) located in the regulatory regions of target genes. The last step in the pathway of receptor action is down-regulation of receptor expression. This down-regulation can be mediated at both the transcriptional levels where the receptor represses new transcription and at the post-translational level where receptor protein is degraded.

The activity of many transcription factors is regulated by phosphorylation or dephosphorylation, and the steroid receptors appear to be regulated similarly. The receptors for glucocorticoids, androgens, progestins, and estrogens are all phosphorylated predominantly on serine residues, although there is evidence that ER is phosphorylated on tyrosine as well (31). Phosphotryptic peptide analysis suggests that human ER is phosphorylated on a single tryptic peptide, and dephosphorylation of this site results in decreased affinity for estrogen response elements [EREs (32)]. These data implicate phosphorylation in the activation of ER's DNA binding activity (33). It therefore seems likely that various activators of cellular phosphorylation cascades, such as those involved in growth factor signaling, could ultimately have an effect on receptor function by regulating its phosphorylation.

While it is well known that estrogen induces several growth factors and receptors [reviewed in (34-36)], it is also apparent that some of these growth factor signaling systems can in turn affect ER function. Dopamine, which activates adenylate cyclase, has been shown to cause estrogen-independent activation of ER (37), and is able to alter the anti-estrogen activity of tamoxifen but not the pure antiestrogen, ICI 164,384 (38). In the rat uterus, where ER and EGFR are expressed in the same cell, EGF can result in the same effects on uterine tract growth and differentiation as estrogen (39), and this effect can be decreased by pretreatment with the pure antiestrogen ICI

164,384 (40) suggesting that these effects are ER mediated (39). EGF or TGF-α can also activate ER transcriptional activity in endometrial adenocarcinoma cells and ovarian adenocarcinoma cells (41). MAP kinase, a downstream effector of growth factor signaling, has been shown to phosphorylate and activate ER in *in vitro* co-transfection experiments of MAPK and ER into ER negative cells. Treatment with EGF of ER-negative cells transiently transfected with ER also activate ER (42).

Experiments performed in ER+ breast cancer cells, however, result in conflicting evidence. Agents that increase intracellular cAMP, such as IGF-I, cause increased rat uterine and MCF-7 breast cancer cell progesterone receptor levels (43,44), as well as activation of transiently transfected reporter constructs containing EREs (45,46), in a manner similar to that of estrogen. In addition to increasing transcriptional activity of ER, ER phosphorylation was increased in these studies (45). This type of interaction between IGF-1 and ER signaling corresponds to the physiologic relationship between these two in MCF-7 cells: that is IGF-1 synerigizes with estrogen in inducing growth (47-51). The transfection of HRG/erbB-2 into MCF-7 cells has also been shown to result in ligand-independent activation of ER (23). However, other HRG transfection studies resulted in down-regulation of ER expression without transcriptional activation (25,52,53). In addition treatment of MCF-7 cells with EGF results in down-regulation of ER without induction of progesterone receptor (54), as does treatment with a novel 52 Kd form of HRG (55). These data suggest that signaling via these pathways in ER+ breast cancer cells can induce the downregulation of ER expression without ligand-independent activation which corresponds to the clinical observation that ER expression is inversely correlated with EGFR or erbB-2 overexpression. Since ER function is

regulated at some level by phosphorylation, these data collectively suggest that several activators of cellular phosphorylation cascades ultimately can effect ER activity and/or expression. If ER is really capable of being regulated by growth factor systems, ligand-independent activation of ER via phosphorylation cascades could represent an early step in the progression of breast cancer from an estrogen-dependent/ER+ phenotype to an ER+ but estrogen-independent intermediate, with subsequent progression to an ER-negative phenotype. Alternatively, upregulated growth factor signaling could induce the ER-negative phenotype by down-regulating ER expression without inducing transcriptional activation.

Many key intermediates in receptor tyrosine kinase signaling have been identified and characterized (56-59). Upon binding of ligand, the activated receptor interacts with and activates the GTP-binding protein Ras and results in the initiation of a phosphorylation cascade involving Raf -- a proto-oncogene that is a serine/threonine kinase, MEK (or MAPKK) -- a dual kinase that is able to phosphorylate both serine/threonine and tyrosine, and MAPK (mitogen activated protein kinase) -- a switch kinase that is activated by phosphorylation on threonine and tyrosine but is itself a serine/threonine kinase. Activated MAPK phosphorylates several nuclear transcription factors including Myc, the TCF protein ELK1 which is responsible for activation of the c-Fos promoter and Rsk, and in this manner effects alterations in cellular proliferation (60-65). MAPK has also been shown to phosphorylate the tumor suppressor p53 although the consequence of this phosphorylation is unknown (66), and as mentioned above, MAPK has also been shown to phosphorylate ER (42). The MAPKs designated as ERKs (extracellular regulated kinases) in this pathway, ERK1, ERK2, and ERK3. Other MAPKs have been recently discovered, although they

seem to participate in parallel but alternate signaling pathways, as discussed below. It is now known that not only can the activated Ras oncogene cause cellular transformation(67,68), but that the activated v-Raf or a constitutively activated MEK can also result in transformation(69-72). Furthermore, a recent study examining the role of the MAPK pathway in renal cell carcinoma found that constitutive activation of MAPK was present in ~50% of their samples, and that this constitutive activation correlated with hyper-activation of Raf-1 and MEK in these samples (73). And most recently, it has been shown in a small, preliminary study that MAPK is both overexpressed and hyperactivated in breast tumors compared to benign breast tissue (74).These data implicate each of these effectors and this specific pathway not only in the transformation process of cells *in vitro* but also in the human carcinogenesis process. While the activity of these factors in breast cancer has not been fully determined, it is reasonable to hypothesize that activation of this pathway also plays an important role in the growth factor signaling involved in progression of breast cancer, and due to interactions with the ER signaling pathway, might play a role in the acquisition of the ER-negative phenotype.

As discussed above, a second class of MAPKs has been identified. These MAPKs mediate the effects of cellular stress activators, protein synthesis inhibitors, cytokines, and some growth factors, and are not activated by the Raf/MEK pathway [reviewed in (59,75)]. In a parallel pathway, a kinase termed MEKK (76-78), which like Raf acts downstream of Ras, activates a kinase called SEK or JNKK (79)which occupies the same position in this pathway as MEK does in the other. MEKK is unlike Raf in two aspects. First, overexpression of a constitutively active MEKK has been shown to result in growth inhibition (77,80). Second, the activation of MEKK

by Ras is not a direct one. Mammalian Rac, which regulates membrane ruffling and Cdc42Hs, which is involved in polarized cell growth, are members of the Rho GTPase subfamily, which acts to regulate the actin cytoskeleton. Rac and Cdc42Hs are intermediate members of the pathway (81-83); that is they act downstream of Ras and upstream of MEKK. The addition of another level of regulation might result in different effectors being able to activate the same pathway. The new MAPKs are referred to as JNKs or SAPKs, which stands for Jun kinase or stress activated protein kinase, and phosphorylate Jun on Ser^{63} and Ser^{73} thereby activating its activity (84-86). JNKs can also activate ELK-1, and thus can activate the same substrates as ERKs but in response to different stimuli (87,88). Because of the different known activators of this pathway, it is associated with both cell growth and growth arrest, and it has been suggested it may play a role in inducing apoptosis.

A third signaling pathway activated by Ras is the PI-3K (phosphoinositide-3 kinase) pathway. PI-3K is activated by tyrosine kinase signaling via Ras in response to a variety of growth factors [reviewed in (89-91)]. In addition to phosphorylating inositol at the 3-OH position, PI-3K is involved in many other cellular processes such as cytoskeleton rearrangement and vesicular trafficking. Activated PI-3K can activate the Raf/MEK/MAPK pathway and the JNK pathway (92,93). One key downstream effector of PI-3K that is not associated with either of these other pathways is Akt (also known as protein kinase B) (94,95). Akt is a serine/threonine kinase and is involved in cell survival. One mechanism for Akt induced survival involves the phosphorylation of the pro-apototic BAD protein. BAD is inactivated by phosphorylation and Akt phosphorylates BAD at Ser136 (96-98). The importance of PI-3K signaling in cancer is highlighted by the recent observation that the alternately spliced EGFR varient, EGFRvIII, which is commonly found in

human tumors, results in constitutive activation of PI-3K (99). Recently, an oncogenic form of PI-3K was isolated from a transformed cell (100). An important role for PI-3K signaling in breast cancer is supported by the demonstration that PI-3K is in the initiation of mouse mammary tumors by neu, TGF-α, and heregulin/NDF (101), and that the induction of mitogenesis in MCF-7 cells by IGF-1 requires PI-3K signaling but not MAPK (102). Since Ras is involved in the activation of both of these additional pathways, it may be the interplay between the Raf/MAPK pathway and these other pathways that determines the ultimate effects of growth factor signaling.

Our original hypothesis was that upregulation of growth factor signaling played an important role in the early events involved in progression of breast cancer from estrogen-dependent to estrogen-independent growth. Our results however have led us to now hypothesize that, in addition to providing alternate growth pathways that confer estrogen-independence, acquired up-regulation of growth factor signaling via overexpression of EGFR or c-erbB-2 leads to the ER-negative phenotype by directly repressing ER expression. Our results indicate several interesting features about upregulated growth factor signaling via Raf-1 in relation to estrogen action in breast cancer cells. First, constitutive signaling through Raf-1/MEK/MAPK in MCF-7 cells does not induce ligand-independent activation of ER as has been observed in other cell types. In fact, such signaling activity represses estrogen-induced activation of ER and results in almost complete down-regulation of ER expression. This down-regulation is observed at the levels of protein, message, and transcription, and the very low level of ER that does remain is expressed in the cytoplasm. Combined, this data along with data from others, might explain the inverse correlation of ER expression with growth factor receptors like EGFR observed in breast cancer and suggests that the signaling pathways

initiated by these growth factor receptors plays an active role in mediating the ER-negative phenotype associated with EGFR high expressing cells. Finally, and most intriguing, is the reversibilty of the ER-negative phenotype of our Raf-transfectants via abrogation of Raf-1 signaling through MEK. This data suggests for the first time that there may exist a subpopulation of ER-negative tumors in which the lack of ER expression is not permanent and could be reversed by inhibition of the appropriate downstream effectors. Our system provides an ideal model in which to delineate the mechanisms underlying Raf-1 mediated down-regulation of ER and determine the role that additional signaling pathways activated by tyrosine kinase receptors play in determining ER levels. Alterations during breast cancer progression in the specific signaling pathways utilized may be important in the overall effects on ER expression. An understanding of these mechanisms may provide us with novel targets not to outright kill tumor cells, but rather to allow for re-expression of ER that would then respond to anti-estrogen treatment.

RESULTS

Stable expression of a constitutively active MEK (ΔMEK) results in a similar phenotype as Δraf. In order to determine if all of the Δraf effects we had observed in our transfectants were being mediated via Raf activation of MEK, we established stable transfectants of MCF-7 cells expressing a constitutively active MEK, ΔMEK (72). Twenty-eight clones were expanded in both FBS- and CCS-containing media and analyzed for expression of ΔMEK (the construct contains an HA-tag so all subsequent analyses use an anti-HA antibody). As shown in Figure 1A, only one

clone, MEK 15, was positive for expression. As we had observed with our Δraf transfectants, however, expansion of this clone in CCS resulted in an even higher expression level (Figure 2B). Since we had determined that suppression of Δraf expression in our original transfection was by histone deacetylation and that this could be reversed using the histone deacetylase inhibitor Trichostatin A (TSA), the ΔMEK clones were treated with TSA and re-analyzed by Western blotting for ΔMEK expression. Results are shown for the MEKpool cell line (pooled population of stably transfected clones) and for three additional MEK lines -- MEK 15, MEK 25, and MEK 27 (Figure 2). In all cases where there was no detectable expression of ΔMEK (every cell line except for MEK 15), treatment with TSA resulted in varying levels of ΔMEK expression. And in the MEK 15 cell line which had originally expressed some ΔMEK, this could be further increased to levels similar to that expressed by MEK 15c cells by treatment with TSA. These results indicated that like Δ-raf, expression of a constitutively active MEK in cells growing in the presence of estrogen was not tolerated.

We next assessed the level of apoptosis and ER expression in the MEK clones. We did not find significant levels of apoptosis (Figure 3), which might initially lead one to think that the Δ-raf induced apoptosis that we observed in our original transfectants was not mediated through MEK. However, the fact that the cells in FBS repress ΔMEK expression at a much faster rate than they did Δraf (indicated by the fact that we were at least able to select and expand several Δraf expressing clones in FBS before they started down-regulating Δraf expression whereas we could only obtain one clone that was still expressing ΔMEK by the time they were expanded for analysis) suggests that we were not able to assess apoptosis under the appropriate conditions. This is further supported by

the fact that the clones placed in CCS down-regulated ER expression very rapidly (Figure 4) and we know from our Raf transfectants that the apoptosis requires high expression of Δraf and ER. Figure 4 shows that compared to control transfected cells which are ΔMEK negative and ER positive, both MEK poolc and MEK 15c exhibit cells with strong positivity for ΔMEK and weak-to-no positivity for ER. Thus, we conclude that the phenotype induced by Δraf is mediated via induction of MEK.

Repression of estrogen action is associated with loss of ER expression. In transient transfection assays of Δraf and an ERE-luciferase reporter construct, we had previously observed that rather than inducing ligand-independent activation of ER, Δraf had instead repressed ER activity. Experiments with ΔMEK had given similar results. We then performed the same transient co-transfections of Δraf or ΔMEK, but instead of performing luciferase assays, subjected the cells to double-label immunohistochemistry, where the cells were immunostained for both ER and for the transfected Δraf or ΔMEK (ΔMEK via its HA-tag). Within the same time frame in which repression of estrogen action was observed by luciferase assay, it can be seen that those cells which stain for transfected gene no longer express ER in their nuclei (Figure 5). These data suggest that rather than inducing ligand-independent activation, signaling via Raf and MEK in ER+ breast cancer cells actually induces down-regulation of ER expression via some other mechanism.

Δraf does not repress a transiently transfected ER promoter reporter construct but does result in differential DNase I hypersensitivity patterns on the endogenous gene. Since we had observed that the rate of ER transcription was significantly lower in Raf 14c than in control-transfected cells, we used transient co-transfection assays with an ER promoter/luciferase construct [kindly provided by Ron Weigel (103)] consisting of -3500 to +210 of the ER promoter driving

luciferase and Δraf into MCF-7 cells in the presence or absence of estrogen to determine if repression was occurring through promoter sequences. As shown in Figure 6A, no significant repression of luciferase activity occurred when Δraf was present. To assess whether these results were an artifact of using a transiently transfected gene, we next performed a DNase I hypersensitivity assay of the endogenous ER promoter to examine whether Δraf signaling results in an altered chromatin structure. It is well-established that the binding of transcription factors results in sites that are hypersensitive to DNase I digestion (104). This assay revealed very interesting differences in specific regions of the ER promoter in Raf 14c cells compared to control transfected cells expressing ER. Of particular interest, there is a strong hypersensitive site in the region of +200 in the control cells that is lacking in Raf 14c cells. This region corresponds to sites important for ER expression and is the area containing two binding sites for ERF-1 (103). In addition, Raf 14c cells gained a site in the region of -800 to -600. This corresponds to a region of potential repressor activity (103). Additional alterations were found and these are summarized in Figure 6B. Thus, combined with the transient transfection data, this data suggests that alterations in chromatin structure affecting accessibility of factors as opposed to alterations in the expression of factors themselves is important in Raf-1 mediated down-regulation of ER expression.

Constitutive MEKK1 signaling does not repress ER activity or down-regulate ER expression. Since growth factor signaling via Ras can also involve activation of the MEKK1/SEK/JNK pathway, we next wanted to determine if the down-regulation of ER expression was specific to signaling via Raf/MEK/MAPK or if it was an effect of any Ras-initiated signaling cascade. We obtained a constitutively active MEKK1 (ΔMEKK1) and performed the same transient

transfection assays with this and an ERE-luciferase reporter construct to assess the effects of ΔMEKK1 on ER activity. As shown in Figure 7, constitutive MEKK1 activity does not result in the same repression of ER activity that we had observed with Δraf or ΔMEK. There is increased activation of both the ERE-luciferase and NON-luciferase plasmids with ΔMEKK1, indicating this is not an ER-specific effect but is rather most likely due to ΔMEKK1 activated AP-1 interacting interacting with the MMTV promoter in which the ERE or NON sequences are inserted. Thus, repression of ER activity associated with down-regulation of ER expression is specific to the Raf/MEK/MAPK arm of Ras-initiated pathways. We are currently assessing the effects of the PI-3K signaling pathway, another Ras-initiated pathway, on these same parameters. However, results from stable transfectants of MCF-7 cells with a constitutively active AKT (a downstream effector of PI-3K activity) from a colleague here at Lombardi indicate that this pathway will not down-regulate ER either since these transfectants remain fully responsive to estrogen.

Overall MAPK activity of Δraf transfectants is similar to that of ER-negative cells. Because we obtained such dramatic effects on ER expression in our Δraf transfectants, we were interested in how the levels of Raf-1 signaling via MEK and MAPK compared between them and several ER-negative breast cancer cell lines with upregulated growth factor signaling components such as EGFR or c-erbB-2. Shown in Figure 8 is a Western blot of whole cell lysates prepared from HCopool, Raf 14c, and several ER-negative breast cancer cell lines probed with an antibody specific only for the phosphoryated, active form of MAPK. As can be seen, Raf 14c cells express significantly more activated MAPK than the control transfected cells, as expected, and all of the ER-negative cell lines have similar or even higher levels of activated MAPK indicating that our

transfectants are not outside the clinically relevant range of MAPK activity. Of particular interest, however, is the relative expression levels of the two different MAPKs, p42 (ERK2) and p44 (ERK1). Strikingly, all of the ER-negative cells predominate in expression of p42, while the ER-positive control transfectants predominate in p44. Raf 14c cells increase expression of both forms, with p42 activity increasing to levels seen in the ER- cells lines. Since it appears that p44 and p42 may signal through different substrates(105), there is the potential for differential expression relating to ER status.

MCF-7/c-erbB-2 and MCF-7/EGFR stable transfectants also have decreased ER expression. The EGFR overexpressing cell line, MCE5 (21), is estrogen independent and exhibits only a very slight reduction in ER expression when grown in estrogen depleted conditions, while MCF-7s over-expressing both EGFR and TGF-α have a further reduction in ER . The c-erbB-2 overexpressing line, MB3 (22), displays constitutively active c-erbB-2 activity, estrogen-independence and tamoxifen-resistance, and a significant reduction in ER levels, while a second clone in which the c-erbB-2 is not constitutively active expresses normal levels of ER . And the FGF-1 overexpressing cell line, α18, is also both estrogen-independent and tamoxifen-resistant, but retains full ER expression (106). I originally performed the ER analyses for these 3 cell lines and I have summarized this data in Figure 9. These results indicate that activation of overexpressed EGFR or c-erbB-2 results in significant repression of ER expression but overexpressed FGF-1 does not.

We have also examined these cell lines for ER activity using the same transient co-transfections of ERE-luciferase or NON-luciferase previously described. MCE5s retain full estrogen

responsiveness and may exhibit a very small amount of ligand independent activation of ER. MB3s, as expected based on ER expression level, exhibit a very blunted estrogen response, and α18s seem unaffected (Figure 10). Since the MB3s express a constitutively active c-erbB-2 and the MCE5s majority of EGFR is not activated, we determined the effect of EGF treatment on the estrogen response of MCE5s (Figure 11). The addition of EGF significantly repressed estrogen induced activation.

Down-regulation of ER expression correlates with level of MAPK activity. Given that we had determined that it was the Raf/MEK/MAPK arm of growth factor initiated signaling that was responsible for down-regulation of ER, we wanted to correlate the ER levels of these transfectants with the their level of MAPK activity. Western blotting using a phospho-specific anti-MAPK antibody were performed to assess the MAPK activity. MB3s had a significant increase in MAPK activity over control-transfected cells, and exhibited a higher overall level of MAPK activity than either of the other two transfected cell lines, MCE5 and α18 (Figure 12A). In fact, α18s exhibited the lowest level of the transfectants. When MCE5 cells were treated with EGF to activate the receptor, a significant increase in MAPK activity was observed (Figure 12B). Collectively, these data suggest that high levels of receptor activation, not just overexpression, is required for down-regulation of ER expression, and that it may be the extent of MAPK activity that determines the level of down-regulation.

Abrogation of Raf signaling by a MEK inhibitor results in re-expression of ER. We had originally demonstrated that loss of ER expression in the Δraf transfectants was reversible by down-regulation Δraf expression via growth in FBS-containing media. Because the loss of Δraf expression

in FBS requires passaging the cells for a period of time, it is possible that other culture-induced changes or selection is occurring. In addition, Raf 14c cells do not appreciably down-regulate Δraf under these conditions making it difficult to re-express ER. Finally, what we are ultimately interested in is repressing the activity not the expression of Raf. To assess the effect of transient shut down of Δ-raf signaling on ER expression, we used the MEK-specific inhibitor, PD 98059, to suppress the activity of this Raf effector. Treated cells were then analyzed for ER expression by IHC. Untreated cells are ER-negative, however, ER is re-expressed in approximately 60% of the cells after 12 hours of MEK inhibition (Figure 13). The staining intensity indicates that ER is expressed at a relatively high level as demonstrated in the magnified view, but not every cell returns to ER-positivity during the time frame of treatment. Thus the abrogation of Raf signaling via MEK and MAPK is sufficient to allow for re-expression of ER.

Construction of a TAT/Δraf fusion protein for protein transduction into cells.

Finally, for many of the experiments we would like to do next, we are interested in the immediate effects of Δraf activity on ER expression. In the application, we had originally planned to use an inducible expression plasmid for Δraf but we have previously explained our problems with getting a system which gives no basal expression but significant levels of induced expression. In addition, it has become clear from experiments described both in last year's report, as well as here, that it is important that we are able to measure the immediate early responses of Δraf **signaling**. Use of a plasmid system necessitates that the protein be made before signaling effects can be measured and in our transient transfection assays, the 24 hours that are necessary to have translated Δraf are also sufficient to see fully down-regulated ER expression. Therefore, we feel that we need the Δraf

protein introduced into cells so that we can then measure the effects immediately. One way we had planned on looking at this question was by first using MEK inhibitor to abrogate Δraf signaling until ER expression returned, and then releasing the cells from MEK inhibition and doing very short time course experiments. This is obviously a very laborious and manipulative way to look at these issues but at the time it was the only way possible to assess the immediate or very early effects of Raf activity on ER expression. Very recently, we have obtained a protein transducing system from Dr. Steve Dowdy. The basis of this system is the creation of a TAT-protein of interest fusion protein. The TAT protein from HIV moves through the plasma membrane into the cell and as fusion protein with a protein of interest (in our case Δraf) will carry that protein into the cell. The key issues with this system is that the protein needs to be unfolded (high ΔG) to cross the plasma membrane efficiently and that the protein is refolded and fully functional within 15-20 minutes. Dr. Dowdy's lab has successfully used this method to study p16, p53, as well as many other proteins. To date, we have created the TAT-Δraf fusion construct and are currently screening bacteria for high producers of the protein. The ability to actually place Δraf protein directly into the cell will give us a second way to study the early effects of Δraf on several parameters of ER expression.

DISCUSSION

Our overall goal for this project was to further our understanding of the mechanisms underlying progression of breast cancer from estrogen-dependent to estrogen-independent growth. It is this acquisition of estrogen-independent growth that moves the cell along a pathway leading to

anti-estrogen resistance or worse, to an ER-negative status. Upregulation of growth factor receptors is a common phenomenon in breast cancer cells and it was our hypothesis that this upregulation is an early event in breast cancer progression. This upregulation would provide estrogen-independent growth signals either by activating ER in the absence of estrogen (ligand-independent activation) or by bypassing ER altogether. Furthermore, the mechanism by which this estrogen-independent growth occurred would be important in assessing anti-estrogen resistance. For example, if growth factor induced estrogen-independent growth was via ligand-independent activation, one would predict that while this may also result in tamoxifen-resistance since tamoxifen would behave like an agonist in this context, pure anti-estrogens such as ICI 182,780 (clinically known as Fasolodex) would not be affected in a such a manner and would thus have therapeutic activity. However, if the ER pathway were being bypassed altogether, even the pure anti-estrogens would be ineffective in inhibiting growth. We were interested in setting up a model that would allow us to study the mechanisms underlying growth factor mediated estrogen-independence.

Raf is a downstream effector of growth factor receptors, such as EGFR or c-erbB-2, whose overexpression is either inversely correlated with ER expression or directly correlated with decreased sensitivity to anti-estrogens (6,7,107,108). Therefore, we have been using the expression of constitutively activated Raf as a model of upregulated growth factor signaling to study the interaction of the growth factor and estrogen receptor mediated signaling systems in the progression of breast cancer from estrogen-dependent growth to estrogen-independent growth. We have previously found that the expression of a constitutively active form of the Raf-1 kinase in MCF-7 ER+, estrogen-dependent human breast cancer cells results in two seemingly linked phenomena.

First, it induced estrogen-independent growth of these cells, both anchorage-dependent and anchorage-independent (27). Second, the expression of Δraf was not tolerated by the cells when grown in the presence of estrogen. In determining the effect of Raf signaling on ER activity, we found that constitutive signaling through Raf-1/MEK/MAPK in MCF-7 cells does not induce ligand-independent activation of ER as has been observed in other cell types. In fact, such signaling activity represses estrogen-induced activation of ER and results in almost complete down-regulation of ER expression. This down-regulation is observed at the levels of protein, message, and transcription, and the very low level of ER that does remain is expressed in the cytoplasm. Combined, this data along with data from others, might explain the inverse correlation of ER expression with growth factor receptors like EGFR observed in breast cancer and suggests that the signaling pathways initiated by these growth factor receptors plays an active role in mediating the ER-negative phenotype associated with EGFR high expressing cells.

In this report, we describe experiments indicating that the Raf-mediated down-regulation of ER is occurring via activation of MEK. Stable transfections of MCF-7 cells with a constitutively active MEK construct resulted in a very similar phenotype as the Δraf transfectants. That is ER was being down-regulated by ΔMEK (Figure 4). We also show that the down-regulation of ER by either Δraf or ΔMEK is relatively rapid as the effect is easily observable in transient transfection assays followed by double-label immunohistochemistry (Figure 5).

In dissecting the downstream signaling effectors responsible for down-regulating ER expression, we found that ER-negative breast cancer cells predominate in ERK 2 activity while ER-positive cells predominate in ERK 1 activity (Figure 8). Since these two ERKs have some different

substrates, we are currently investigating the possibility that it is an ERK 2 specific substrate that mediates down-regulation of ER. Secondly, in examining changes in protein interaction with the ER promoter, we have found that both loss of a specific enhancer binding site as well as acquisition of two putative repressor binding sites correlated with the lack of ER expression in our Δraf transfectants (Figure 6). In addition, we used this same Dnase I hypersensitivity assay to analyze the ER promoter in BT474 human breast cancer cells. These cells are interesting because they are ER+ with higher levels of both EGFR and c-erbB-2 than most ER+ cells but the ER levels are decreased in these cells. We were interested in determining if the ER promoter in these cells displayed an intermediate pattern between that for control transfected ER+ cells and Δraf transfected ER- cells. In preliminary experiments, we found that these cells have lost the specific enhancer binding site but have not gained the putative repressor binding sites. Thus, we have identified an intermediate pattern in cells with decreased but still present ER. We are currently performing fine mapping experiments to determine the sequence of the putative repressor binding sites.

Given these very interesting data, we wanted to confirm the physiological relevance of our Δraf signaling. We therefore assessed the levels of MAPK activity in our Δraf transfectants compared to several other ER- breast cancer cells lines with known upregulation of growth factor signaling pathways. Even our clone with the highest Δraf levels and thus the highest MAPK activation levels was comparable to the panel of breast cancer cells lines examined (Figure 8). In some of these other ER- cell lines, the MAPK activity was even higher than that of our Δraf transfectant. We next wanted to determine if the down-regulation of ER expression mediated by upregulated growth factor receptor activity was specific to signaling via the Raf/MEK/MAPK

pathway or if other Ras-initiated effector pathways could also result in this phenotype. We performed transient transfection assays with a constitutively active MEKK1, the equivalent of Raf-1 in the MEKK/SEK/JNK pathway, and observed no significant effect on ER activity (Figure 7). Another pathway activated by Raf is the PI-3K pathway and stable transfectants of a constitutively AKT, a downstream effector of PI-3K signaling, are still fully responsive to estrogen indicating that ER is not down-regulated in these cells. We therefore conclude that it is the Raf/MEK/MAPK arm of growth factor induced signaling that is responsible for the down-regulation of ER expression.

We then wanted to correlate our results with MCF-7 cells transfected with specific growth factor receptors or growth factors. The rationale for this was that each of these transfectants exhibited differential effects on ER expression (Figure 9). The data suggested that it wasn't just a matter of growth factor/receptor overexpression but rather hyperactivation that correlated with down-regulation of ER expression. This data correlates well then with our Δraf and MEK transfectants which essentially are reflections of hyperactivation of this signaling pathway. Furthermore, in assessing the level of MAPK activation among the different transfections it was apparent that only very high levels of MAPK activity, as mediated by EGF activation of overexpressed EGFR or by a constitutively active c-erbB-2 (Figure 12), resulted in down-regulation of ER expression. This further supports the idea that it is the Raf/MEK/MAPK arm of growth factor signaling that is responsible for ER down-regulation and further suggests that it is the extent of MAPK activity that determines the extent of down-regulation.

Finally, and most intriguing, is the reversibilty of the ER-negative phenotype of our Raf-transfectants via abrogation of Raf-1 signaling through MEK. This data suggests for the first time

that there may exist a subpopulation of ER-negative tumors in which the lack of ER expression is not permanent and could be reversed by inhibition of the appropriate downstream effectors. Our system has provided an ideal model in which to start delineating the mechanisms underlying growth factor signaling mediated down-regulation of ER and determine the role that specific signaling pathways activated by tyrosine kinase receptors play in determining ER levels. Alterations during breast cancer progression in the specific signaling pathways utilized may be important in the overall effects on ER expression. An understanding of these mechanisms may provide us with novel targets not to outright kill tumor cells, but rather to allow for re-expression of ER that would then respond to anti-estrogen treatment.

Material and Methods

Cell Culture. MCF-7 human breast cancer cells were obtained from the Lombardi Tissue Culture Core Facility (originally from Marvin Rich, Michigan Cancer Foundation), and were maintained in Improved Minimal Essential Medium (IMEM, Gibco/BRL,) with phenol red supplemented with 10% fetal bovine serum (FBS, Intergen Company). For growth in the absence of estrogen, media was switched to IMEM supplemented with 10% charcoal stripped calf serum (CCS, Gibco/BRL). For certain experiments, cells were completely stripped of estrogen by repeated rinsing of cells in IMEM and growth in IMEM + 10% CCS (once per day for 4 days) or in others, they were quick-stripped (3 times per day for 2 days). Hormone treatments, when performed, were with 17β-estradiol (Sigma) at 10^{-8} M or the pure antiestrogen, ICI 182,780 (obtained from Alan Wakeling, Zeneca Pharmaceuticals) at 10^{-7} M. Cells were plated in CoStar (Cambridge, MA) 75 cm^2 T-flasks

and grown in a forced air humidified incubator at an atmosphere of 5% CO_2 and 37° C.

Stable Transfection with ΔMEK. The ΔMEK construct was obtained from Dr. Natalie Ahn; it has two substitutions (Ser-218 and Ser-222 have been replaced with acidic residues) and a truncation from residues 32 to 51 (a suspected kinase regulation domain) which result in approximately 400 times the activity of the wild-type MEK (72). Expression is driven by a CMV promoter and a nine amino acid long influenza hemagglutinan (HA) tag is located at the amino terminus to allow for its detection separately from the endogenous, wild-type MEK. The pCHC6 plasmid described in our original report contains a hygromycin resistance gene driven by CMV and this was co-transfected with ΔMEK. As a control for transfection, the pCHC6 plasmid was used alone. Transfections were performed using the Chen and Okayama method of calcium phosphate transfection. Briefly, 20 μg of ΔMEK plasmid DNA and 10 μg of pCHC6 were incubated in 0.25M Ca_2Cl and 2X BES-buffered saline for 10 min at room temperature (rt). The DNA solution was incubated with MCF-7 cells (at ~70% confluence in 10 cm tissue culture dishes) for ~ 16 hrs at 35° C and 2% CO_2. The cells were rinsed twice with serum-free IMEM and then re-fed with IMEM + 10% FBS. After a 48 hr recovery, the cells were split 1:5, allowed to plate for 24 hrs, and then selected in FBS media containing 150 μg hygromycin B (Boehringer Mannheim). Individual colonies were selected using trypsin-soaked filter paper disks which were placed in 24-well plates in FBS media. Clones from each transfection were expanded and analyzed for expression of the transfected MEK.

Gel Electrophoresis and Western Blotting. For detection of ΔMEK, cell lysates were prepared as described previously (27). Briefly, cells were rinsed in PBS and then lysed in a modified Gold Lysis Buffer [20 mM Tris, pH 7.9, 137 mM NaCl, 5 mM EDTA, 10 % glycerol, 1% Triton X-100, 1 mM

EGTA, 1 mM Pef-bloc A (instead of PMSF), 1 mM aprotinin, 1 mM leupeptin, 1 μM pepstatin A, 1 mM bacitracin, 1 mM Na_3VO_4, 1 mM sodium pyrophosphate, 10 mM soduim flouride] on ice, scraped into a microfuge tube, and centrifuged at 12,000 xg to pellet nuclear debris. Supernatants were analyzed for protein content using the BCA protein assay kit (Pierce) and stored at -20^0 C. 100 μg of cellular lysate was electrophoresed through 10% SDS-polyacrylamide gels as above. Electrophoresed gels were transferred for 2 hrs at 0.4 amps in Towbin's buffer (20 mM Tris, 150 mM glycine, pH 8.3, 20% methanol, 0.1% SDS), and the blots were blocked in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) with 10% nonfat dried milk. Blots were then incubated with an anti-HA tag antibody for detection of ΔMEK diluted 1:1000 in TBST plus 1% nonfat dried milk overnight at 4° C. After washing the blots 3 X 5 min with TBST, they were incubated with goat anti-mouse antiserum linked to horseradish peroxidase (BioRad) diluted 1:4000 in TBST/1% nonfat dried milk for 60 min at rt. The blots were again washed in TBST, once for 20 min and then 3 X 5 min, and the bound secondary antibody visualized using enhanced chemiluminescence (Pierce) according to manufacturer's instructions. For detection of P-MAPK or MAPK, blots were blocked in TBST with 3% BSA and an additional 0.15% tween-20 overnight at 4° C. Blots were then incubated with an anti-PMAPK polyclonal antibody that specifically recognizes only the phosphorylated form of MAPK or with an anti-MAPK polyclonal antibody that recognizes all forms of MAPK (New England Biolabs) diluted 1:1000 in TBST plus 1% BSA overnight at 4° C. Blots were processed as above except the secondary antibody was donkey anti-rabbit antiserum linked to horseradish peroxidase (Amersham) and diluted 1:4000 in TBST/1% BSA for 60 min at rt.

Tunel Assay for Apoptosis. The ApoTag for FACS kit was obtained from Oncor and the assay was

performed according to the kit directions. Control cells for the assay, a human leukemia cell line treated with campothecin for 24 hrs to induce ~30% apoptosis, were obtained from Phoenix Flow Systems as already fixed cells. Floating cells were collected and either combined with trypsinized adherent cells or analyzed separately. Two million cells were fixed for 15 min on ice in 1% paraformaldehyde. Following washes, cells were suspended in ice-cold 70% ethanol and stored at -20°C for less than 2 months prior to being analyzed. Fixed cells were pelleted, washed, suspended in Equilibrium buffer, and incubated with a reaction mix consisting of Tdt and fluorescein-labeled nucleotides for 30 min at 37°C. Cells were then washed, resuspended in Stop buffer, and analyzed using a FACStar^{Plus} laser system (Becton Dickinson).

Transient Transfection and Luciferase assays. MCF-7 cells were plated in Falcon 6-well plates, allowed to attach overnight, and were then quick-stripped of estrogens by repeated washing and replacing of the media with IMEM w/o phenol red supplemented with 10% CCS 3 times per day for two days. At the end of the second day, cells were transfected by the calcium phosphate, low CO_2 protocol. Briefly, each well was transfected with 2.5 μg of luciferase plasmid and 1.0 μg of of Δraf or Δmek plasmid suspended in $CaCl_2$ and mixed with BBS. The luciferase plasmids were either pGLB-MERE or pGLB-MNON, obtained by inserting an MMTV promoter containing either a double consensus ERE (MERE) or the same sequence with the ERE palindromes scrambled (MNON) (13) into the Hind III site of Promega's pGLB basic luciferase plasmid. Other luciferase plasmids used included the ER promoter luciferase construct (103) obtained from Ron Weigle. The cells were incubated for 18 hrs at 2% CO_2 and 35° C, were then washed two times with PBS, and then incubated for 48 hrs in media containing vehicle (0.01 % ethanol), 10^{-8} M estradiol, 10^{-7} M ICI

182,780, 10 ng/ml EGF, or a combination of EGF and estrogen. Cells were assayed for luciferase activity (expressed as relative light units of RLU) using Boehringer Mannheim's kit according to the manufacturers instructions. The luciferase values were normalized for protein to obtain RLU/mg, and the RLU/mg values were adjusted to specific RLU/mg by subtracting out the value obtained with lysate prepared from mock-transfected cells. The duplicates were then averaged, and the values were plotted as specific RLU/mg protein. Or cells were subjected to double-label IHC.

Immunohistochemistry Assay. Cells were plated in 2-well chamber-slides (Falcon), allowed to attach, and grow as a monolayer. For ER expression cells were quick-stripped of estrogen. Cells were fixed by incubation for 10 min at rt with 3.7% formaldehyde-PBS, followed by ice-cold acetone for 15 sec. Fixed cells were then blocked by incubation for 60 min at rt in PBS with 1% BSA. For ER detection, cells required permeabilization by incubation in PBS with 0.1% triton X-100 for 5 min at rt. Primary antibody incubations were overnight at rt in a humidified chamber and were at 2.5 μg/ml for anti-ER and at 0.5 μg/ml for anti-Raf diluted in PBS/1%BSA. The anti-HA antibody used for detection of the HA-tagged ΔMEK was used at 1:1000. After 3 PBS washes, secondary antibody incubations were for 60 min at rt and were a 1:200 dilution of biotinylated anti-rat for ER and a 1:300 dilution of HRP-linked anti-rabbit for Δraf or 1:800 for anti-HA in PBS/1% BSA. Detection of ER required a further incubation of 30 min at rt with streptavidin-alkaline phosphatase (AP) and then visualization with Vector Red (to give a red color). Detection of Δraf or HA-tagged ΔMEK required just visualization with Vector SG (to give a blue/gray color). For double IHC, both primary antibodies were incubated together, followed by both secondaries together, visualization of Δraf or HA-tagged ΔMEK, and then streptavidin-AP and visualization of ER. Stained cells were then

dehydrated through a graded series of ethanol, followed by xylene, and mounted in permount. All incubations were followed by three washes of 30 sec each and no counterstain was used.

DNase I Hypersensitivity Assay. Nuclei were isolated by suspension of cells (approximately 1-2 x 10^8 cells per experiment) in 20 ml of reticulocyte standard buffer (RSB; 10mM Tris-HCl, pH 7.4/10mM NaCl/3mM $MgCl_2$) containing 0.5% Nonidet P-40. The nuclei were washed several times in RSB and resuspended at a concentration of approximately 3 x 10^7 nuclei per ml in RSB. 300µl aliquots of nuclei were digested with DNase I (Sigma) at concentrations ranging from 0.1 to 32 µg/ml for 10 min at 37°C. Controls were an aliquot of nuclei with no DNase I added, kept on ice for 10 min. Digestion was stopped by the addition of EDTA to 25mM, SDS to 0.5%, and proteinase K to 0.5 mg/ml, followed by incubation at 37°C for 5 hrs.

DNA was purified by two rounds of extraction with phenol/chloroform, one with chloroform, and ethanol precipitation. The samples were then treated with RNase A, and the extractions and precipitation were repeated. Samples of purified DNA were digested with Xba I according to the recommendations of the supplier. For agarose gels, 12 µg of each sample were electrophoresed and alkaline transferred to a charged nylon membrane (Hybond N+, Amersham). The ER probe (a Xba I/Bam HI fragment from the 5' promoter sequence was labeled by random oligonucleotide priming, and hybridization was carried out for 20 hrs at 65°C in 5X SSPE (1X SSPE: 180mM NaCl/10 mM$NaPO_4$, pH 8.3, 1mM EDTA)/0.02% Ficoll/0.02% polyvinylpyrrolidone/0.01% BSA/0.2% SDS/100 µg/ml herring testes DNA/10% Dextran Sulfate. After hybridization, filters were washed three times for 30 min with 5mM $NaPO_4$, pH 7.0/1mM EDTA/0.2% SDS at room temperature, once

for 30 min with 0.1X SSC (1X SSC: 150mM NaCl/15mM sodium citrate, pH 7.3)/0.1% SDS at 60°C, and once for 30 min with 0.1X SSC/0.1% SDS at 65°C, prior to autoradiography at -70°C with Kodak XAR-5 film and DuPont Lightning Plus intensifying screens.

ER Steroid-Binding Assay. Whole cell extracts were prepared as described above for ER Western blotting. Extracts were incubated with 10 nM [^{3}H]-17β-estradiol plus or minus a 100-fold excess of unlabeled estradiol for 16 hrs at 4° C. Binding was assayed using the DCC (dextran-coated charcoal) assay as described previously (109). In short, the DCC was added to adsorb free hormone, and was then pelleted by centrifugation. Aliquots of supernatant were removed and counted in 10 mls of liquid scintillation fluid in a Beckman liquid scintillation counter.

MEK Inhibition. Inhibition of MEK activity was accomplished using PD 98059 [a Parke-Davis compound obtained from New England Biolabs (NEB)]. PD 98059 was resuspended in DMSO according to the manufacturers instructions. A one hour treatment with 50 μM has been shown to effectively suppress the MEK induction of MAPK activity in response to various growth factors in other cell systems (as described in Product Data Sheet from NEB). However, it is suggested that much higher doses are necessary if strong or sustained signaling is occurring. The conditions for abrogating Δraf signaling in Raf 14c cells using PD 98059 were determined by analysis of phosphorylated MAPK expression as a downstream consequence of Raf signaling via MEK. Because Δraf results in constitutive signaling, a higher dose for a longer time was required to fully suppress signaling. It was determined that a 200 μM dose for three hours was required to completely abrogate activation of MAPK in Raf 14c cells. After 4 hours, some return of phospho-MAPK was even observed with the 200 μM dose. Therefore, cells were treated four times consecutively with

the 200 μM dose for three hours to a total of twelve hours of complete MEK inhibition. Cells were then washed in PBS and processed for immunohistochemistry.

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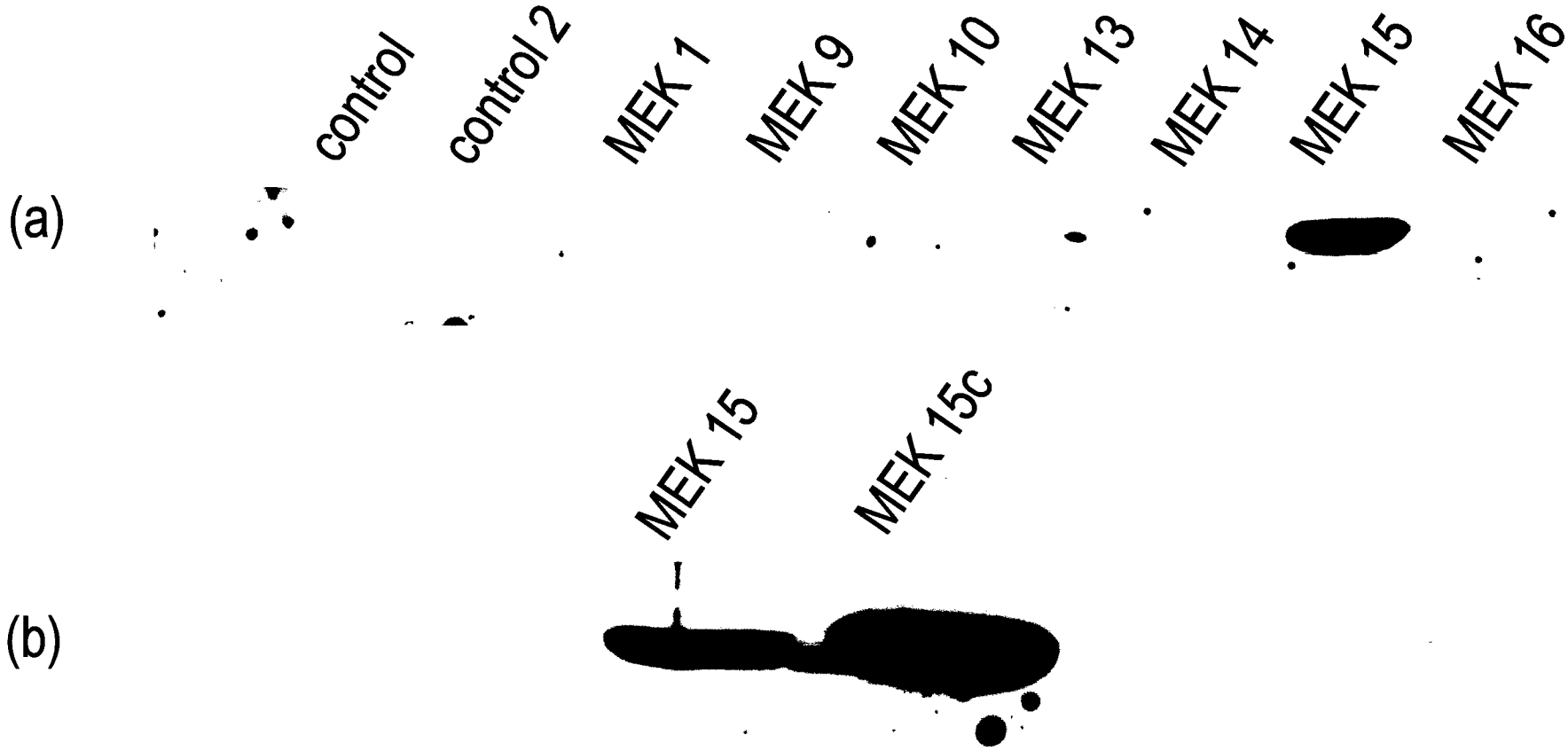


Figure 1 Identification of positive clones by Western blotting for HA-tag. Lysates were made after initial expansion of clones. Either (a) 50μg or (b) 100μg of whole lysate was electrophoresed through a 10% polyacrylamide gel. The proteins were then transferred and probed with anti-HA antibody (Berkeley). a. MEK 15 shows itself to be the only clone expressing Δ-MEK, which runs at a molecular weight of just above 46K. b. The MEK 15c represents MEK 15 cells transferred to and grown in non-estrogenic medium after the initial expansion of the clone. The lysate was made after 2 passages in the non-estrogenic medium.

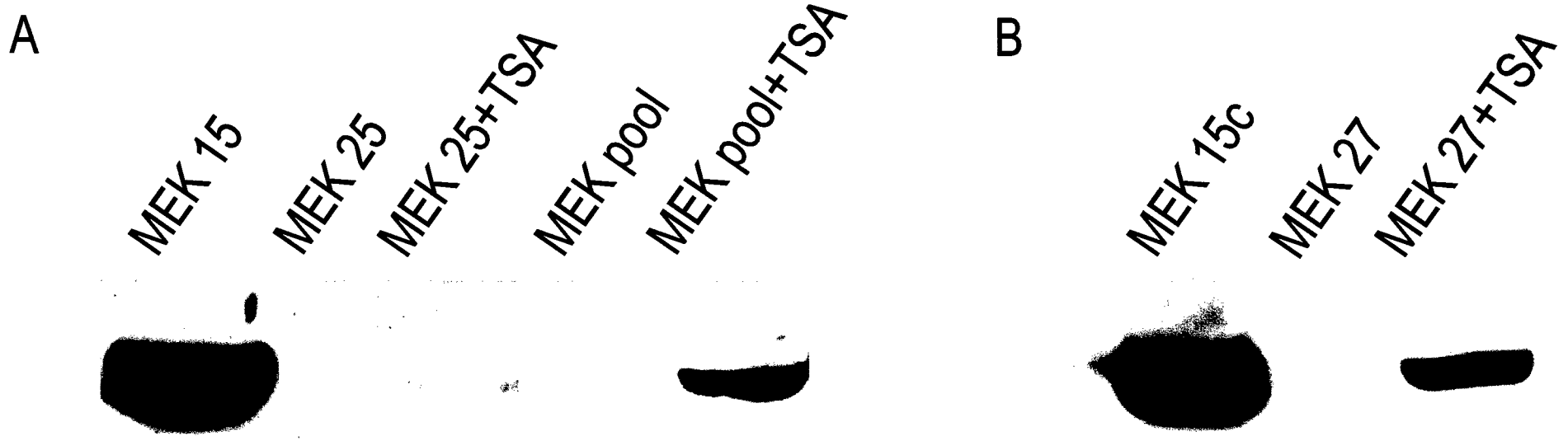


Figure 2 Treatment with trichostatin A results in re-expression of Δ-MEK. Cells were were treated with 3μM trichostatin A for 24 hrs, lysates prepared, and either (a) 100μg or (b) 70μg of protein was electro-phoresed through a 10% polyacrylamide gel. Proteins were then transferred and probed with anti-HA tag antibody(Berkeley). +TSA indicates cells treated with trichostatin A, and MEK15 or MEK 15c were used as postive controls.

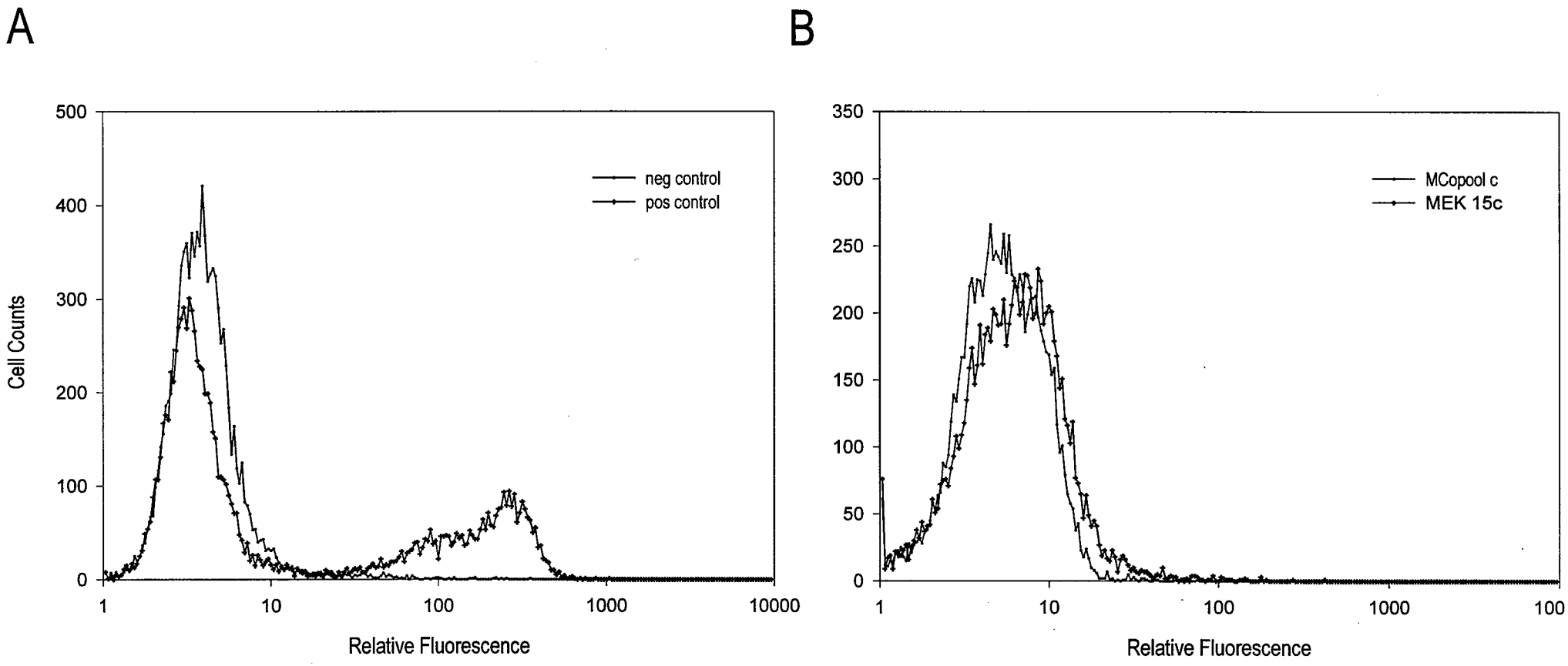


Figure 3 Induction of apoptosis by DMEK. Determination of relative apoptosis was done via the TUNEL assay using the Apotag kit. A. Positive and negative control cells. B. MCopoolc and MEK 15c cells.

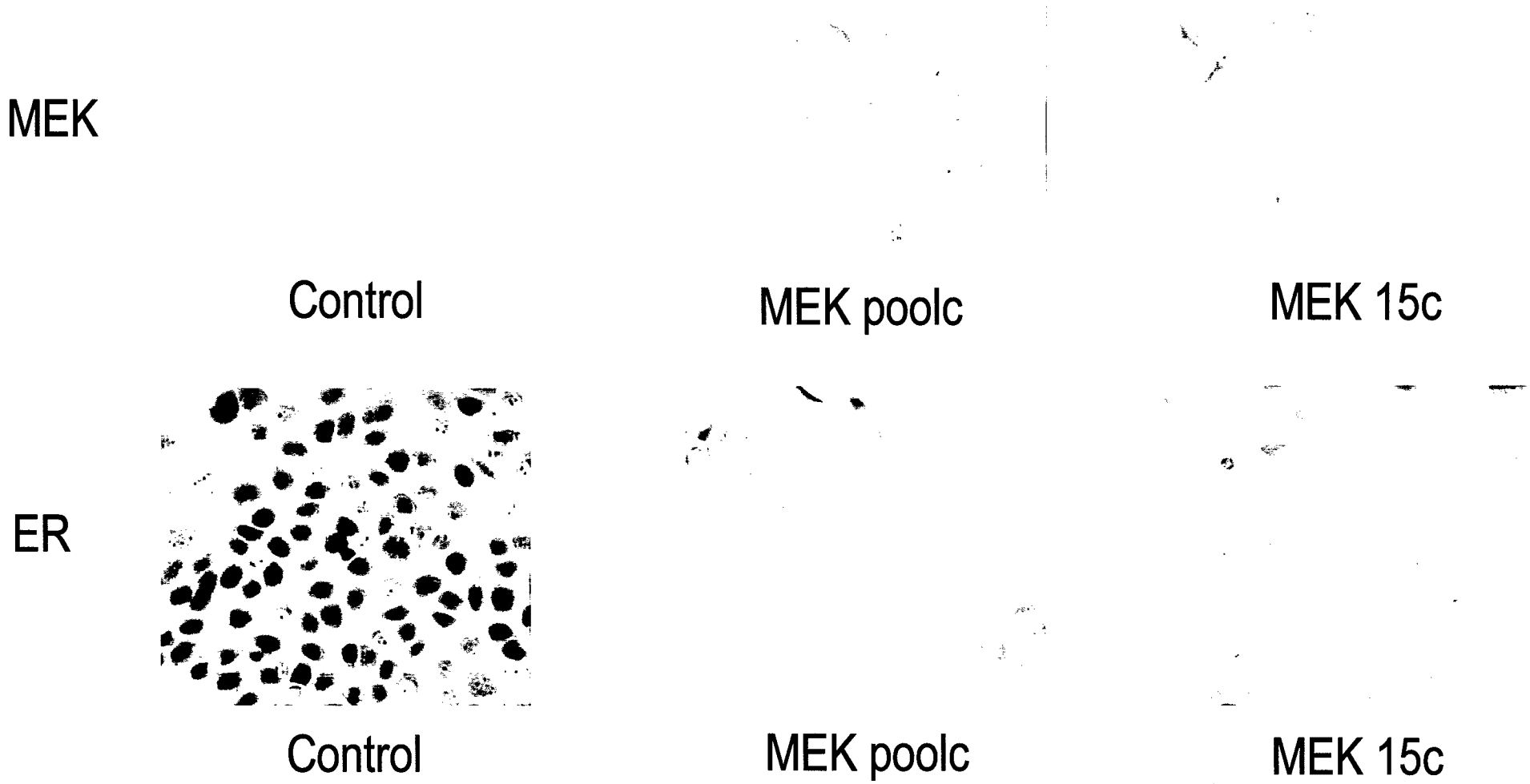
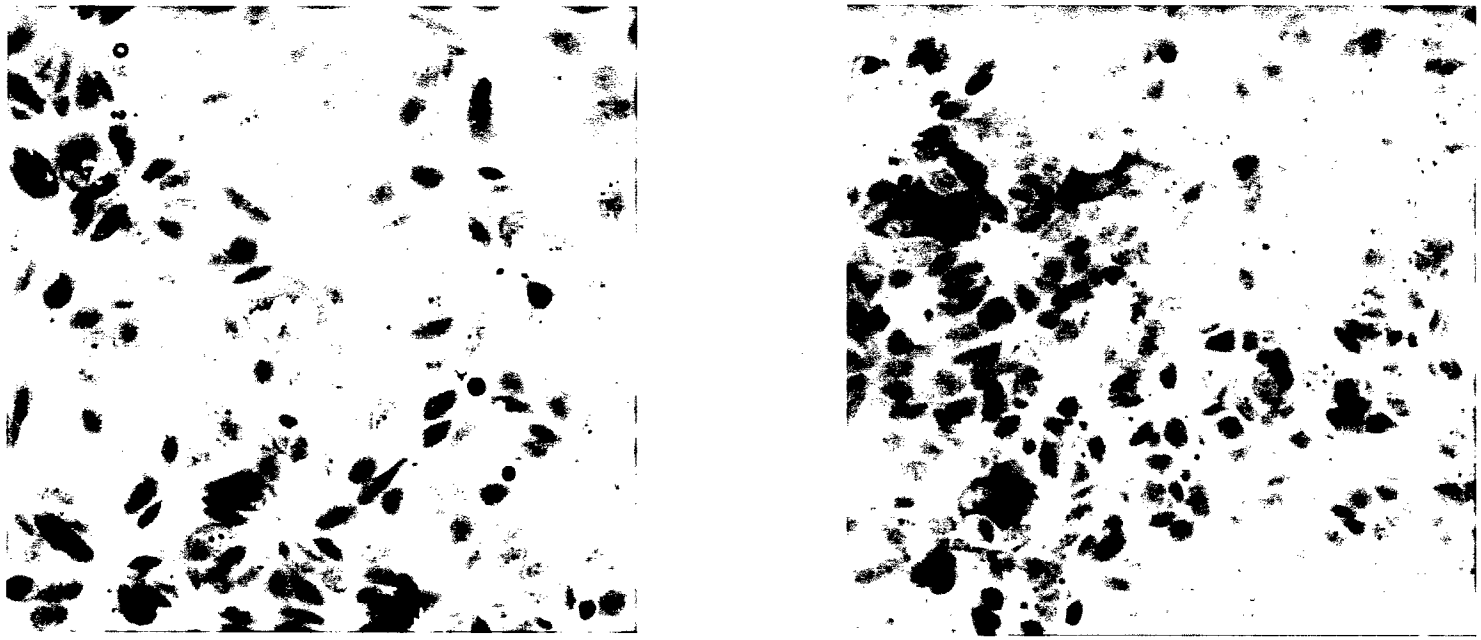


Figure 4 Stable expression of ΔMEK leads to reduced ER levels. Top panels are stained for MEK (blue-gray stain). Bottom panels are stained for ER (red stain).

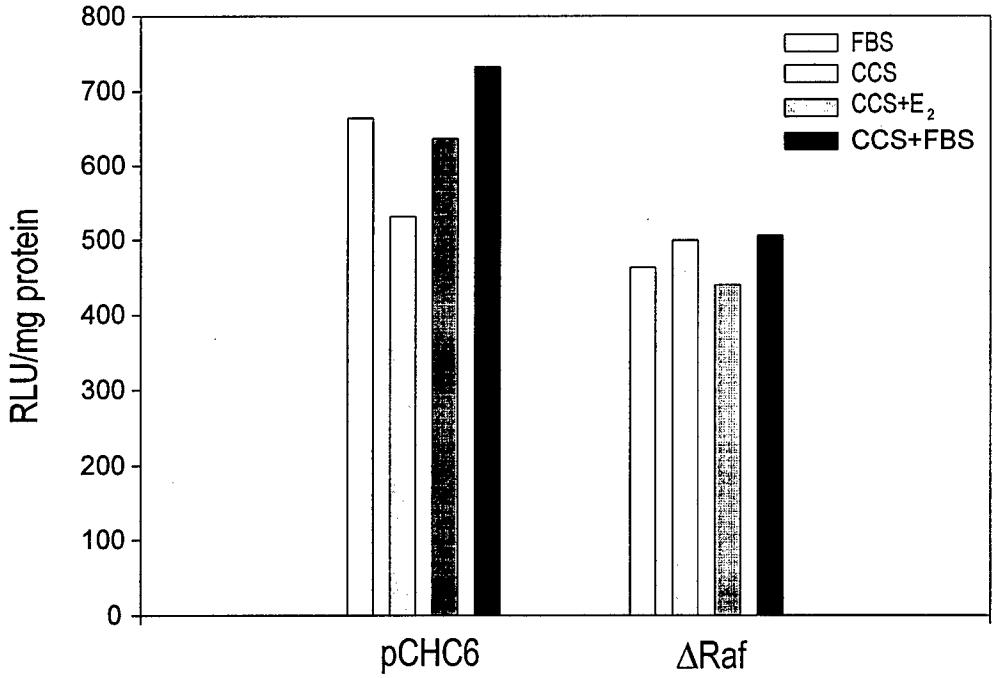


ΔRaf

ΔMEK

Figure 5 Transient expression of Δraf or ΔMEK results in down-regulation of ER. MCF-7 cells were transfected and analyzed by double- label IHC for ER and Δraf (grey staining) expression or ER and ΔMEK (grey staining) expression. Right panel is a magnified view (400X) of image in left panel (100X).

A



B

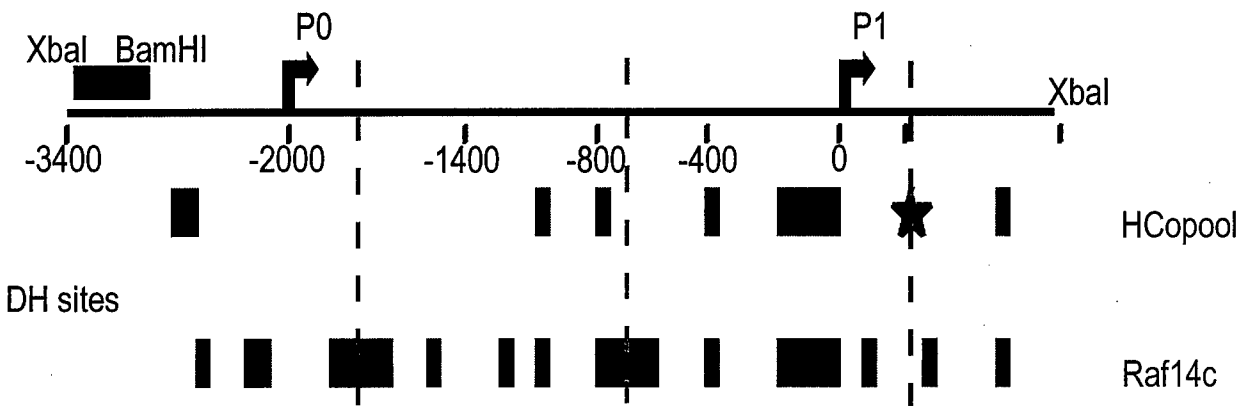


Figure 6 Δraf effects on the ER promoter. (a) Δraf was co-transfected into MCF-7 cells with an ER promoter luciferase construct, in FBS, CCS, or CCS +10-8 M estradiol. (b) Identification of regions in the ER promotor that are hypersensitive to DNase I in HCopool and Raf 14c cells. Nuclei were treated with increasing concentrations of DNase I, genomic DNA was isolated, digested with XbaI, Southern blotted, and probed with the indicated XbaI-BamHI fragment (shaded box). Position 0 is the start of transcription from P1, the solid boxes indicate DH sites, and the * indicates a very strong site in control cells

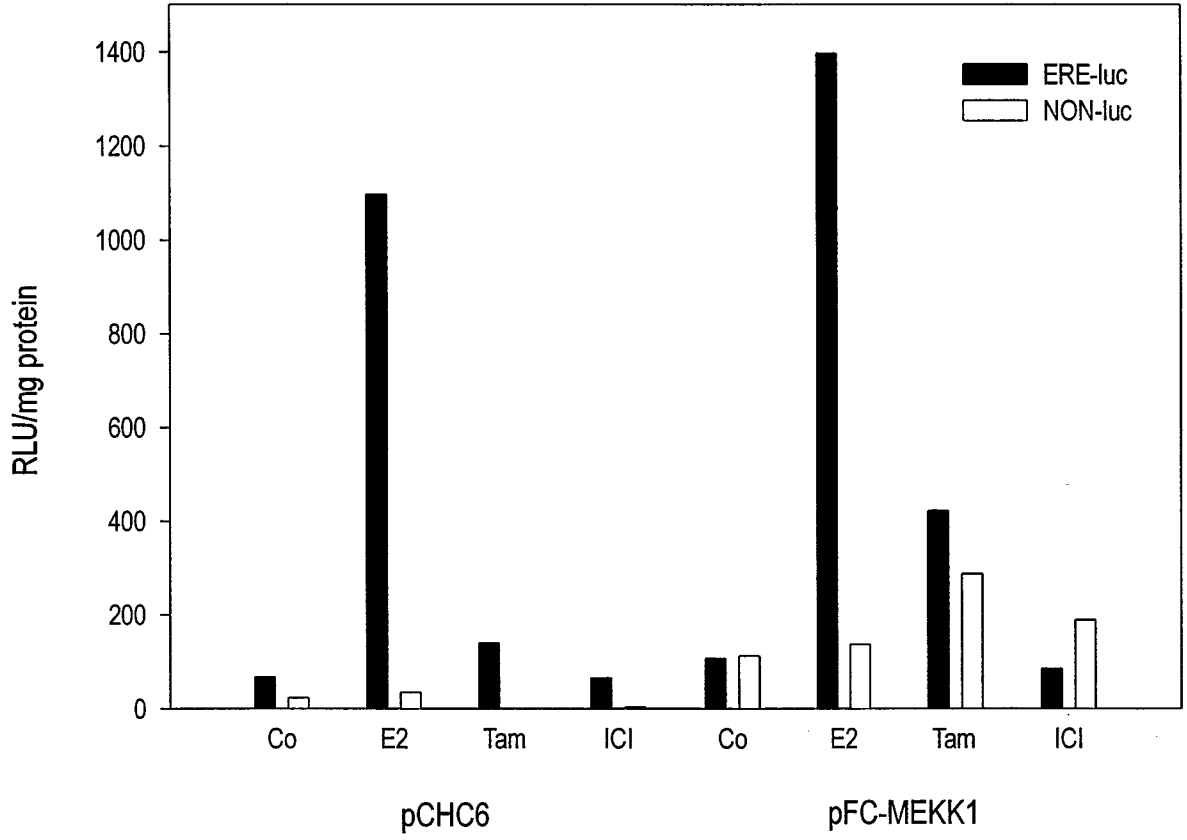


Figure 7 MEKK1 signaling does not repress estrogen action. MCF-7 cells quick-stripped of estrogens were transfected with 2.5 µg of ΔMEKK1 and either ERE-luc or NON-luc. Post-transfection treatments were for 48 hrs in media plus or minus 10-8 M estradiol, 10-7 M 4-OH tamoxifen, 10-7 M ICI 182,780.

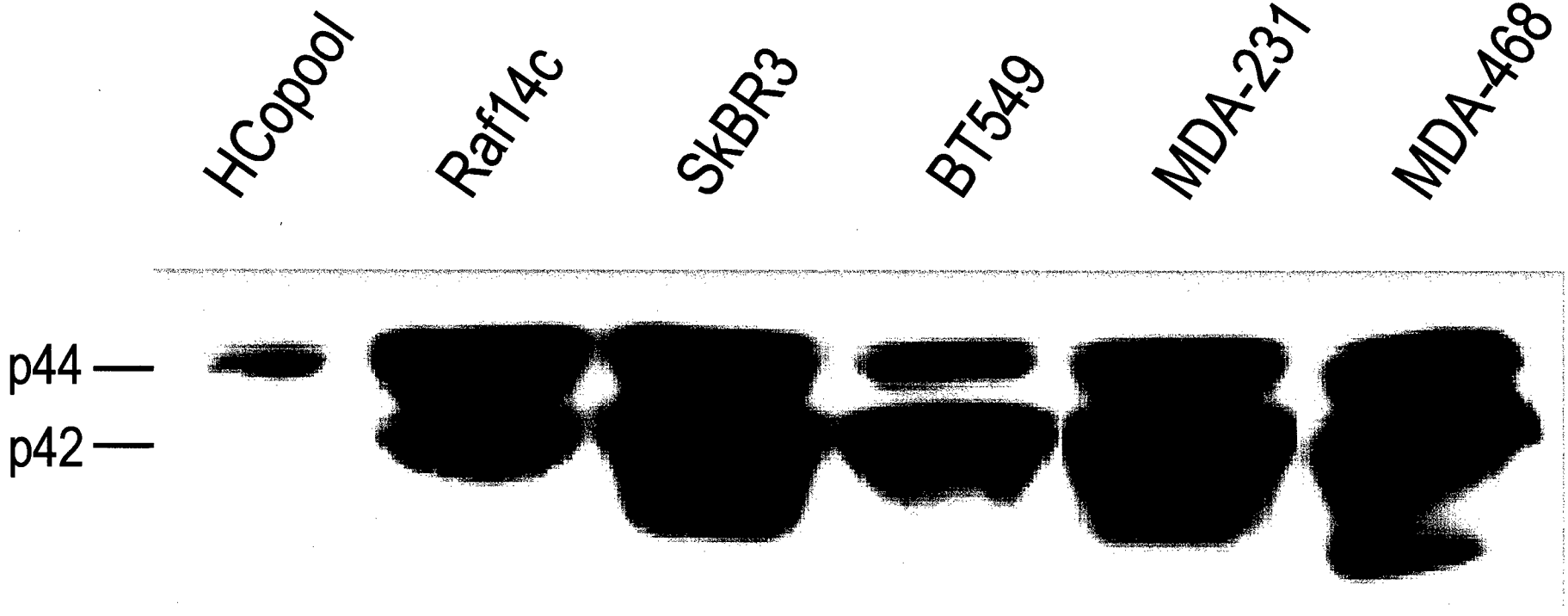


Figure 8 Phospho-MAPK expression in a panel of breast cancer cell lines. Whole cell lysates were analyzed for MAPK activity by Western blotting with an anti-phospho MAPK antibody (from NEB) that only recognizes the phosphorylated, activated form of MAPK.

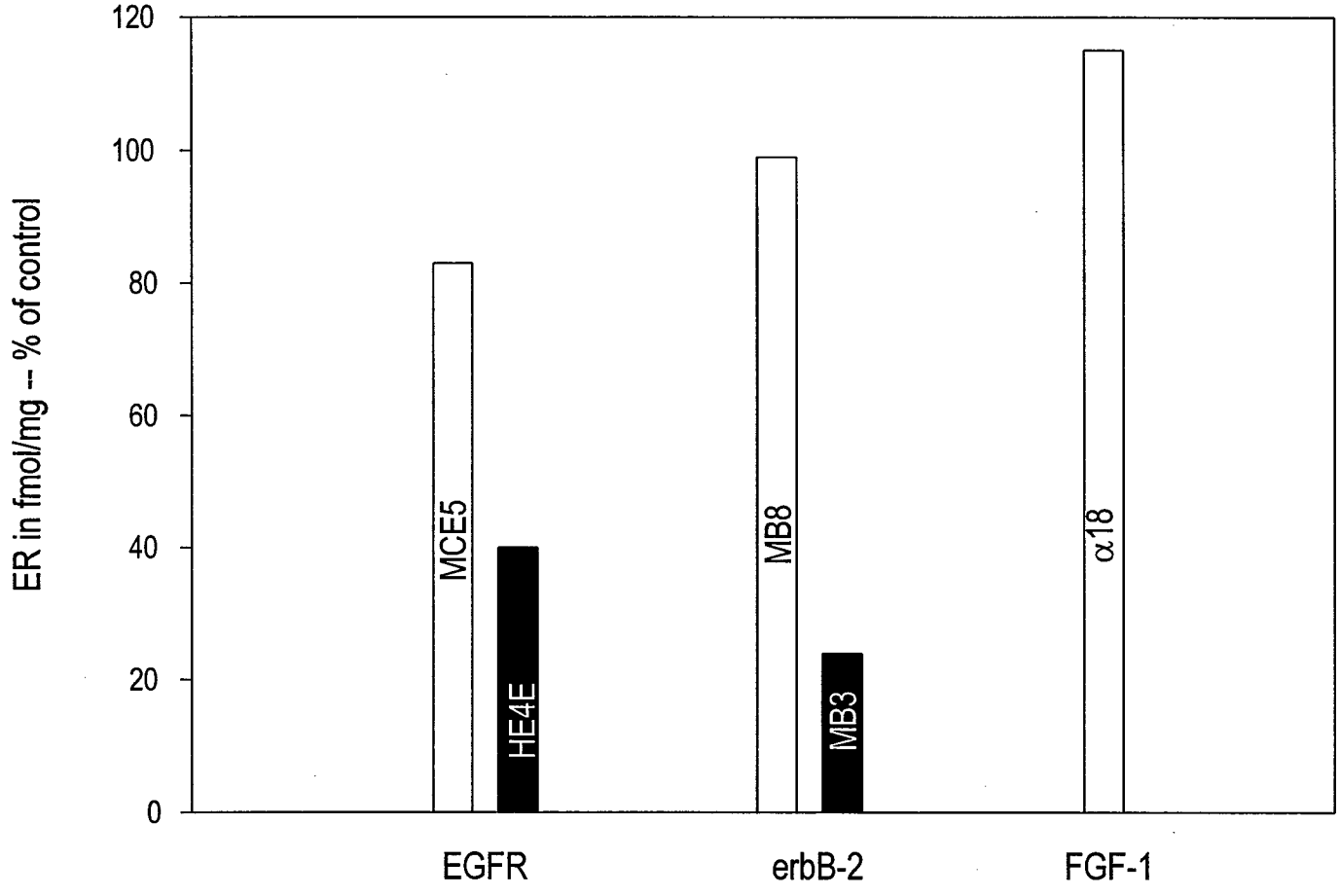


Figure 9 ER Levels in MCE5s (EGFR overexpressing), HE4Es (EGFR and TGF-α overexpressing), MB8s (c-erbB-2 overexpressing), MB3s (constitutively active c-erbB-2 overexpressing), and α18s. ER was measured by the DCC steroid-binding assay as described. Results are expressed as % of ER in fmol/mg protein of control MCF-7 cells.

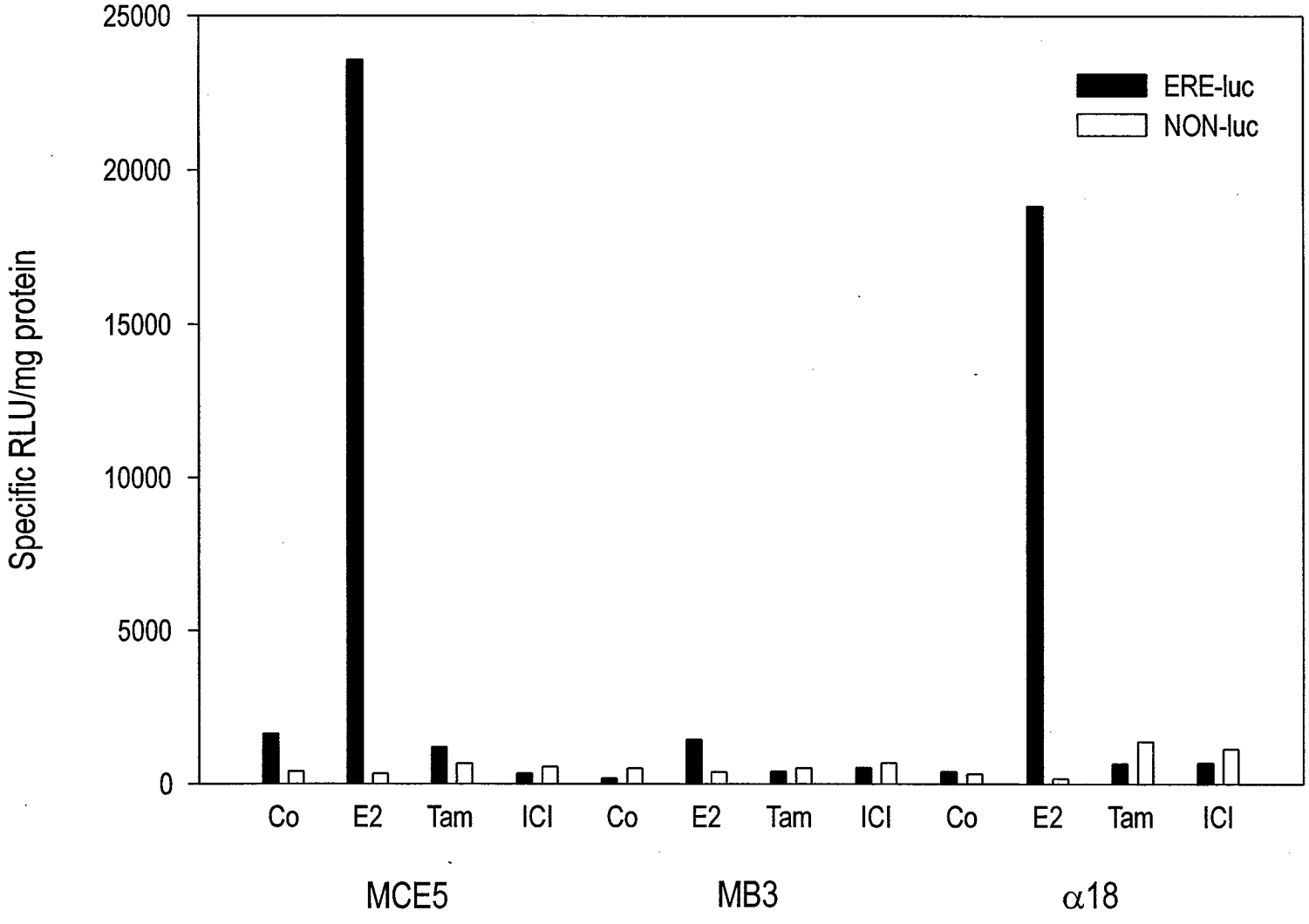


Figure 10 Constitutive c-erbB-2 signaling represses estrogen action. MCE5, MB3, or α18 cells were transfected with 2.5 µg of ΔMEKK1 and either ERE-luc or NON-luc. Post-transfection treatments were for 48 hrs in media plus or minus 10-8 M estradiol, 10-7 M 4-OH tamoxifen, 10-7 M ICI 182,780.

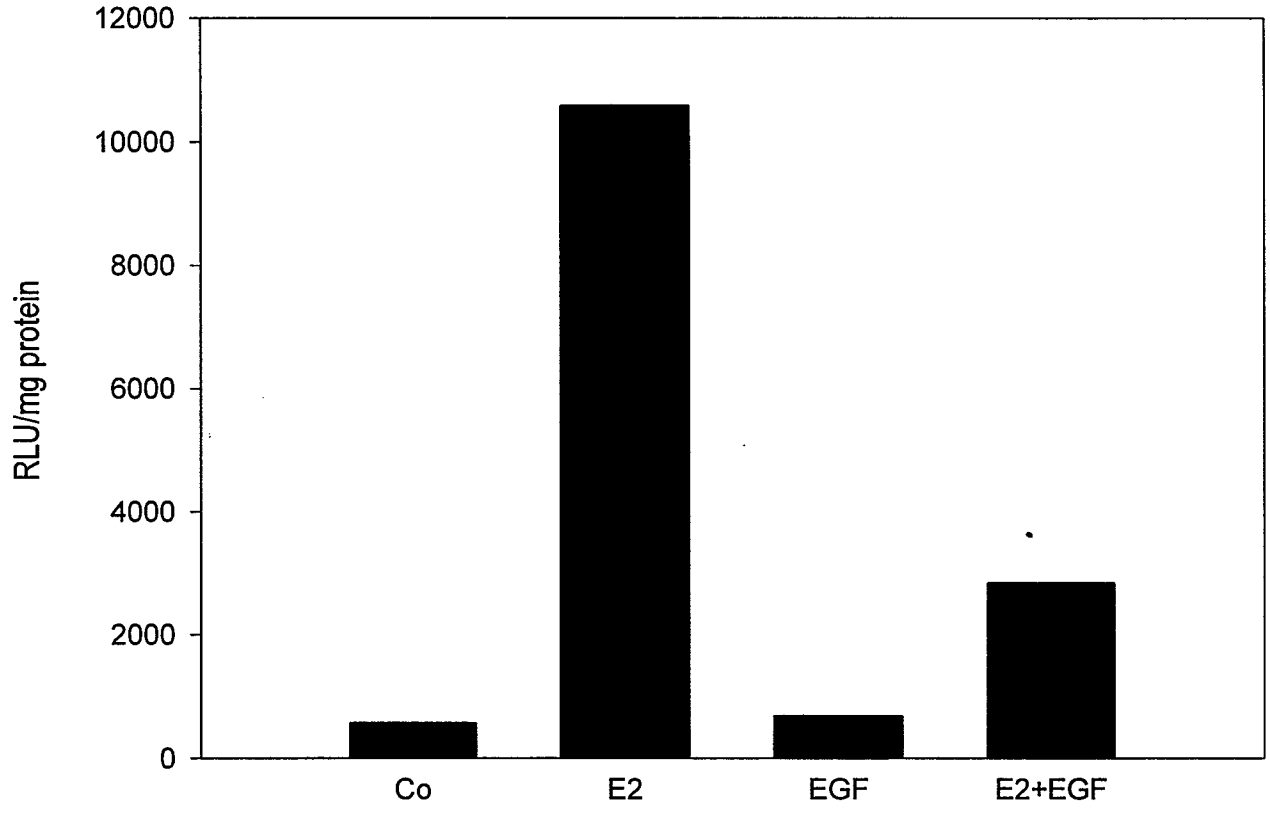


Figure 11 High EGFR signaling represses estrogen action. MCE5 cells were transfected with 2.5 µg of either ERE-luc or NON-luc. Post-transfection treatments were for 48 hrs in media plus or minus 10-8 M estradiol or plus or minus 10 ng/ml EGF as indicated.

Figure 12 Phospho-MAPK expression in MCF-7 growth factor receptor/growth factor transfectants. Whole cell lysates were analyzed for MAPK activity by Western blotting with an anti-phospho MAPK antibody (from NEB) that only recognizes the phosphorylated, activated form of MAPK. In (B), MCE5 cell were treated with 10 ng/ml EGF for 15 min.

Co 12 hr 12 hr-mag

Figure 13 Transient abrogation of the Δraf signaling via the MAPK pathway in Raf 14c cells results in re-expression of ER. Cells were treated with 200μM MEK inhibitor, PD98059, every 3 hours for a total of 12 hours. Cells were then immunostained for ER expression. The right panel represents a higher magnification (400X) of the middle panel (100X).

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REPLY TO
ATTENTION OF:

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